FINAL PROGRESS REPORT

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PRINCIPAL INVESTIGATOR: Dr. Peter T. Lansbury, Jr.

INSTITUTION: Massachusetts Institute of Technology

GRANT TITLE: Models of Glycoprotein Folding

AWARD PERIOD: 15 November 1988-30 November 1991

OBJECTIVE: To develop a convergent method of glycopeptide synthesis, and to investigate the influence of attached carbohydrate on peptide structure.

APPROACH: We have completed the development of a synthetic method. This method is summarized in the attached paper which has been submitted to the *Journal of the American Chemical Society*. Complex glycopeptides have been synthesized and are awaiting structural characterization.

ACCOMPLISHMENTS (for the 3 year period): A synthetic method has been developed. This method is summarized in the attached manuscript which has been submitted to the *Journal of the American Chemical Society* for publication. Structural studies involving solution NMR are underway.

SIGNIFICANCE: Availability of synthetic glycopeptides is a necessary prerequisite for structural and other studies of this class of compounds. This technique allow glycopeptides to be made in an efficient, convergent approach, which introduces the precious and relatively unstable carbohydrate in a late step. The mild final deprotection should not harm the sugar at all, even for the most sensitive glycoside linkages.

PUBLICATIONS:

1. A Convergent Approach to the Chemical Synthesis of Asparagine-Linked Glycopeptides. Shimon T. Anisfeld and Peter T. Lansbury, Jr. *J. Org. Chem.*, **1990**, *55*, 5560.

2. A Practical Convergent Approach fo Glycopeptide Synthesis. Shimon T. Anisfeld-Cohen, and Peter T. Lansbury, Jr., manuscript submitted to *J. Am. Chem. Soc.*.

PATENTS: None

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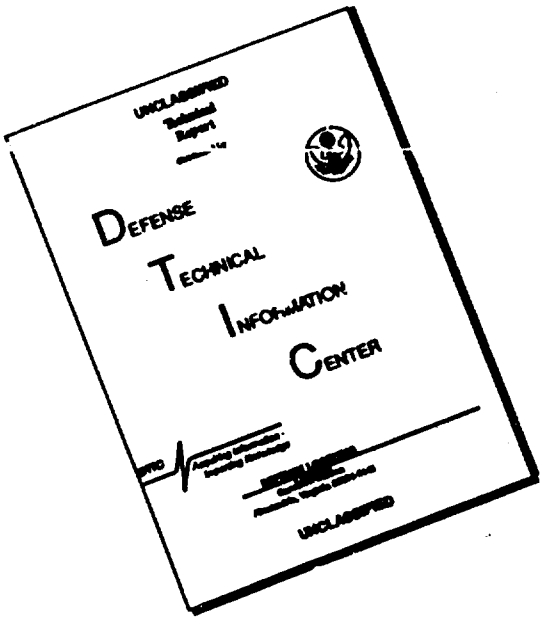

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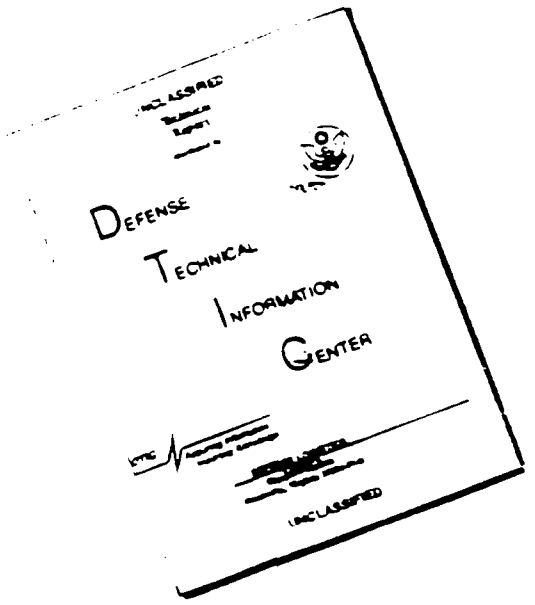

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This research involves the synthesis and analysis of models of glycoprotein folding. These models are glycopeptides. Glycopeptides are extremely difficult to synthesize using existing technology. Therefore, the bulk of our effort has been expended in an effort to develop a practical method for the synthesis of these molecules. The method that we have developed is simple, uses commercially available materials and is extremely economical. We have used this method to prepare complex glycopeptides which are derived from natural glycoprotein structures. The analysis of these materials is underway using solution NMR techniques.

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PATENTS: None

A Practical, Convergent Method for Glycopeptide Synthesis.

Shimon T. Cohen-Anisfeld[1] and Peter T. Lansbury, Jr.*

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ABSTRACT

Glycopeptides are useful compounds to model the conformational effects of the biosynthetic glycosylation of asparagine (N) residues in glycoproteins. We report herein a practical, convergent method for the synthesis of N-glycopeptides. The key reaction involves the acetylation of a β glycosyl amine with a partially protected peptide. Commercially-available protected amino acids and peptide synthesis resin are used. The β glycosyl amine can be derived from any reducing sugar by a simple procedure. Optimized experimental protocols are included for each step. Several glycosylations involving complex and acid-sensitive oligosaccharides are reported, including the coupling of a heptasaccharide (**8**) with a pentapeptide (**14**) in 55% purified yield.

INTRODUCTION

Many secreted and cell-surface proteins are modified by the covalent attachment of carbohydrate to an asparagine (Asn,N) residue via a β-N-glycosidic linkage (Figure 1).[2] The structures of N-linked oligosaccharides fall into three basic types: high-mannose, complex, and hybrid oligosaccharides. All of these contain the common pentasaccharide core $(Man)_3(GlcNAc)_2$, but differ in the nature of the outer residues.[3-5] The effects of these sugars both on the folding of the protein and on its final structure constitute areas of significant interest.[4, 6-10] N-Glycopeptides are often used as models for studying these interactions,[11-15] and therefore a convenient route to these compounds would be of great value.

insert Figure 1 here

Synthesis of N-glycopeptides has been carried out most often by the stepwise approach, in which a glycosyl amine is coupled to a suitably protected Asp derivative to give an Asn(Sug) derivative, which is then deprotected and elongated to give the desired glycopeptide.[16-18] There are several solid-phase methods available which utilize this approach.[19-25] These methods suffer from two major disadvantages related to the introduction of the sugar at an early stage in the synthesis. First, some of the O-glycosidic bonds present in complex oligosaccharides are not completely stable to the acidolytic deprotection conditions normally used in peptide synthesis. Although the methods mentioned above have been designed to minimize the exposure of the glycosidic bonds to acid, they all require a trifluoracetic acid (TFA) treatment step for resin cleavage or side chain deprotection. While this may be acceptable for certain oligosaccharides, it is likely to lead to O-

glycosidic bond cleavage in some cases, particularly for the more sensitive linkages. Second, the early introduction of the sugar means that several equivalents of sugar are needed and that, because the sugar must survive additional amino acid coupling steps and deprotection, the overall yield from oligosaccharide to glycopeptide is low. This may be acceptable for monosaccharides or for oligosaccharides which are available in large amounts, but may be an impediment to the synthesis of glycopeptides containing complex oligosaccharides which are available from synthetic or natural sources in only small quantities. In the convergent strategy reported herein, the sugar must endure, at the most, two mild deprotection steps.

As an alternative to these methods, we have proposed a convergent approach, based on the coupling of the carbohydrate amine to an Asp-containing, partially-protected peptide.[11, 12, 26-29] The introduction of the sugar in a late step requires less material and avoids exposure of the oligosaccharide to acidic conditions. In addition, the convergent approach allows the synthesis of a series of glycopeptides containing different oligosaccharides, without the need to resynthesize the peptide for each individual case.

In order to make the convergent approach a viable alternative to the stepwise strategy, three problems must be solved.[30] First, since glycosylation of a peptide is expected to be slower and more difficult than glycosylation of an amino acid, especially when the sugars are large; a potent coupling reaction is needed in order to carry out a high-yield glycosylation of a peptide. Second, when a peptidyl Asp side chain is activated for glycosylation, there is the potential for a competing, relatively facile intramolecular reaction, namely, cyclization to the succinimide[31-33] (Figure 2); this side reaction must be minimized. Third, a protective group scheme must be developed which allows selective deprotection of one Asp residue, with other

protective groups remaining intact. After the glycosylation, the other protective groups must be removed in a mild manner.

insert Figure 2 here

In a previous paper,[26] we began to deal with the first two of these issues, by reporting the optimization of a simple glycosylation to minimize succinimide formation and maximize yield. The present paper discusses the synthesis of appropriately-protected peptides for glycosylation, as well as the synthesis of the other partner in the glycosylation reaction, the β-glycosyl amine. In addition, the optimization of several complex glycosylation reactions is reported, as well as the utilization of the convergent approach for the synthesis of a variety of glycopeptides, including glycopeptides containing acid-sensitive and precious oligosaccharides, compounds which would be particularly difficult to synthesize by the stepwise approach. The glycopeptides which have been synthesized according to our strategy are among the most complex members of this class yet prepared by chemical synthesis.

RESUME REVISIONS HERE

RESULTS AND DISCUSSION

Synthesis of β-glycosyl amines

β-Glycosyl amines have generally been synthesized by reduction of the corresponding azides.[28, 34-36] More recently, several groups[24, 37-39] have begun to use the much simpler approach introduced by Kochetkov,[40] in which the reducing

oligosaccharide is treated for an extended period of time with saturated aqueous ammonium bicarbonate to afford exclusively the β isomer of the corresponding amine. In addition to the glycosyl amine, the crude product of this reaction usually contains some starting material and, in some cases, side products, such as the diglycosyl amine.[24, 37, 40, 41] Because of the instability of the glycosyl amine, purification is undesirable and thus this crude product has been used directly in glycosylations.[24, 37]

It is important to determine the amount of glycosyl amine present in the crude reaction mixture, especially in the case of precious sugars. In addition, there is a need for a way to confirm that all the NH_3 from the reaction mixture has been removed, since any that remains will produce the undesired Asn-containing peptide. We have developed an HPLC assay which meets both these needs. After workup, a measured amount of the crude glycosyl amine is coupled to a known amount of Boc-Asp-OBn, using 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)[42] as coupling reagent. Quantitation by HPLC of the ratio of Boc-Asn(Sug)-OBn, Boc-Asn-OBn, and Boc-Asp-OBn provides an estimate of the amount of glycosyl amine and the amount of ammonia present in the crude product. This HPLC assay is an indirect measure whose success depends on the efficiency of the glycosylation of Boc-Asp-OBn (we have, in several cases, measured 80-90% conversion to glycosyl amine).

With this analytical method in hand, we set out to prepare several glycosyl amines for glycopeptide synthesis. The desired glycosyl amines are shown in Figure 3. $GlcNAcNH_2$ (**1**) is commercially available. Chitobiose constitutes the disaccharide core of N-linked sugars; the peracetylated compound (**2**) is commercially available. Fucα1-6GlcNAc was of interest to us because the addition

of a fucose to the interior GlcNAc of the core structure is a biological event whose consequences are unclear.[3-5] In addition, the Fucα1-6 linkage is a very acid-sensitive one, so that glycopeptides containing this sugar cannot be easily prepared by non-convergent methods.[43] The peracetylated disaccharide (5) was synthesized from L-fucose and GlcNAc-OBn by a modification[30] of the literature procedure.[44-46] The heptasaccharide $(Man)_5(GlcNAc)_2$ (7), which occurs in many high-mannose glycoproteins, was made available to us by Dr. Christopher Warren of Massachusetts General Hospital. This compound is isolated from the urine of sheep with swainsonine-induced α-mannosidosis, a disease in which the catabolism of mannose-containing glycoproteins is impaired, causing the buildup of mannose-containing oligosaccharides.[47, 48]

insert Figure 3 here

These sugars were converted to the amines using the Kochetkov reaction. In some cases, the peracetylated compounds were treated directly without prior deacetylation, with the expectation that the ammonia would remove the protective groups as well as forming the amine.[23, 37] After a simple workup consisting of drying *in vacuo* to a constant weight (to remove NH_3), the samples were analyzed by the HPLC assay described above. The results are shown in Figure 3. Conversion to glycosyl amine was considerably less efficient in the cases where peracetylated sugar starting materials were used. This may be due to the formation of SugNHAc as a side product. In any case, these results indicate that *peracetylated sugars should be deprotected (e.g., by Zemplen hydrolysis) before use in the Kochetkov amination reaction*. For unprotected sugar starting materials (e.g., **3** and **7**), this reaction provides good yields of glycosyl amines. The β stereochemistry of these amines was confirmed by NMR analysis of the stereochemistry of the sugar-Asn linkage after

glycosylation ($J_{NH,H1}$ ~ 9 Hz); no evidence of the α anomer was found in any of the glycopeptides synthesized.

Synthesis of appropriately protected peptides

In order to synthesize complex glycopeptides, a double-deprotection scheme was required. A first deprotection step, performed after peptide synthesis but before glycosylation, should deprotect the Asp to be glycosylated but leave other Asp residues and other reactive side chains protected (Figure 4, step 2). A second, mild deprotection step, performed after glycosylation, should remove the remaining protective groups (Figure 4, steps 4&5).

In our approach (Figure 4), the peptide is synthesized using Boc amino acids on the methylbenzhydryl amine (MBHA, produces C-terminal peptide amide) resin, which is cleaved by strong acid (HF). Certain residues which are generally protected during peptide synthesis do not need to be protected during glycosylation, and are therefore protected with protective groups which are removed during the cleavage from the resin; these residues are Tyr, Ser, Thr, and Arg (see Figure 4, step 2). The Asp to be glycosylated is likewise protected with an acid-labile group; the cyclohexyl ester (cHex) is preferable to the benzyl ester (Bn), in order to minimize aspartimide formation during peptide synthesis. The other reactive amino acids are protected with groups which are stable to the HF cleavage but which can be removed under mild conditions after glycosylation. For Lys, Glu, and Asp (other than the one to be glycosylated), protective groups are used which can be removed by treatment with piperidine (or the even milder base morpholine), namely, the fluorenylmethoxycarbonyl (Fmoc) carbamate group (for Lys) and the fluorenylmethyl ester (Fm) group (for Glu and Asp). For Cys and His, protective

groups are used which can be removed by treatment with thiophenol, namely, the ethyl disulfide (for Cys) and the dinitrophenyl (DNP) group (for His). All of the protected amino acids required for this scheme are commercially available.

insert Figure 4 here

Several peptides were synthesized using this protection strategy (Figure 5). The peptides Ac-E(Fm)DASK(Fmoc)A-NH_2 (**9**) and Ac-C(SEt)DH(DNP)TRA-NH_2 (**10**) were designed to test this protective group scheme. Ac-AE(Fm)AAAK(Fmoc)E(Fm)AAAK(Fmoc)E(Fm)DASK(Fmoc)A-NH_2 (**11**) and Ac-AE(Fm)AAAK(Fmoc)E(Fm)DASK(Fmoc)E(Fm)AAA-K(Fmoc)A-NH_2 (**12**) have sequences based on the helix-forming peptide of Marqusee and Baldwin,[49-51] and were designed to test the effect of the sugar on the peptide conformation. Ac-E(Fm)E(Fm)K(Fmoc)YDLTSVL-NH_2 (**13**) comprises residues 288-297 of ovalbumin and is glycosylated, in vivo, with a high-mannose type oligosaccharide. These peptides were synthesized on the MBHA resin using the protective groups shown in Figure 4 and cleaved from the resin with HF. In the case of Ac-E(Fm)DASK(Fmoc)A-NH_2, the peptide was purified by HPLC before glycosylation. For the other peptides, the glycosylation was carried out on crude peptide. In addition, the peptide Ac-YDLTS-NH_2 (**14**), comprising residues 291-295 of ovalbumin, was synthesized. Since this peptide contains no residues which require protection during the glycosylation, it was synthesized using a standard peptide synthesis protocol, rather than the approach described above. This peptide was purified by HPLC before glycosylation.

insert Figure 5 here

Table 2

	Peptide	Sugar	$SugNH_2$ (eq.)	DIEA (eq.)	product distribution (%)[a] glycopep.	pep.	imide
1.	**9**	**1**	1	0	30	33	37
2.	**9**	**1**	1	1	31	b	69
3.	**9**	**1**	2	0	55	b	45
4.	**14**	8	2	0	73	25	2
5.	**14**	8	1	1	48	46	6
6.	**14**	8	2	1	81	12	7

[a] product distribution was determined by measurement of HPLC peak heights; [b] peptide was present at ≤5% and was not included in calculating the product distribution.

Table 3

Peptide	Sugar	$SugNH_2$ (eq.)	DIEA (eq.)	HBTU (eq.)	HOBt (eq.)	Yield[a]
9	**1**	2	0	9	5	29% (step 3)[c]
10[b]	**1**	2	1	5	0	43% (step 3)[b], 48% (step 5)[d]
11[b]	**1**	2	0	5	5	11% (steps 3 & 4)[b]
12[b]	**1**	3	0	5	5	12% (steps 3 & 4)[b]
13[b]	**1**	3	0	5	5	39% (steps 3 & 4)[b]
14	**1**	2	0	3	1	61% (step 3)
14	**4**	2	2	3	0	88% (step 3)
14	**6**	1	2	5	5	54% (step 3)
14	**8**	1	2	5	5	55% (step 3)

[a] yield shown is the purified yield for the indicated step(s) (see Figure 4); [b] these peptides were not purified prior to glycosylation. Yields are based on an estimate of peptide purity by amino acid analysis. [c] deprotection (step 4) was quantitative by HPLC; [d] the unprotected glycopeptide was isolated as the disulfide-bonded dimer.

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A Convergent Approach to the Chemical Synthesis of Asparagine-Linked Glycopeptides

Shimon T. Anisfeld and Peter T. Lansbury, Jr.*

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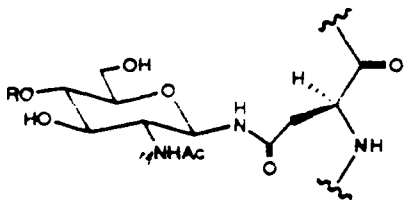
Summary: Reaction conditions for the direct coupling of glycosylamines to aspartic acid containing peptides are described.

A cotranslational process which is common to the biosynthetic pathways of many cell-surface and secreted eukaryotic proteins involves transfer of $(Glc)_3(Man)_9(GlcNAc)_2$ to an asparagine amide nucleophile to produce an asparagine-linked glycoprotein (1).[1] Because it occurs



1 R = $(Glc)_3(Man)_9(GlcNAc)_2$

cotranslocationally, glycosylation may play a role in the folding of glycoproteins.[2] In order to model the interactions between the nascent polypeptide chain and the attached oligosaccharide, we have undertaken the chemical synthesis of a series of glycopeptides. The existing methods of glycopeptide synthesis involve coupling of a glycosylamine to a suitably protected aspartic acid to give a protected asparagine–carbohydrate conjugate, followed by selective deprotection and elaboration of the glycoamino acid in a stepwise manner.[3-10] The O-glycosidic linkage present in complex oligosaccharides is not stable to the acidolytic deprotection conditions normally used in peptide synthesis;[3,6] therefore, application of this strategy to complex targets requires the use of specialized amino acid derivatives[5,10] or the elaboration of the oligosaccharide portion of the glycopeptide by chemical or enzymatic methods. As a practical alternative, we are seeking to develop a convergent approach based on the coupling of an oligosaccharide β-glycosylamine to an aspartic acid containing peptide.[11] Large peptides could be made and purified by standard techniques, followed by the introduction of acid-sensitive and synthetically precious oligosaccharide in a late step. Realization of this strategy requires a high-yield coupling reaction for the formation of the glycopeptide amide bond and a protection/deprotection scheme which allows selective deprotection of the desired aspartic acid and, subsequently, mild deprotection of the product glycopeptide.[3] This paper focuses on the coupling reaction and reports the synthesis of four glycopeptide amides.

While the coupling of protected glycosyl amines (e.g. **2**, Chart I) to α-esters of aspartic acid (Asp) proceeds in good yield,[3,6,7,12] the coupling to Asp-containing peptides may be complicated by competing intramolecular succinimide formation.[13-15] In order to minimize succinimide formation and achieve a high-yield coupling, we have found that several factors must be carefully controlled. The activation of the peptide–aspartate carboxyl group, the minimization of base in the reaction medium, and the choice of protection for the carbohydrate hydroxyls all play critical roles (see Table I). Peptide **4**[15] was chosen for model studies because the valine residue adjacent to Asp was expected to hinder succinimide formation.[13] Activation of peptide **4** with diisopropylcarbodiimide (DIC) did not effect coupling, whereas coupling of the 1-hydroxybenzotriazole

(1) (a) Sharon, N. *TIBS* **1984**, 198. (b) Olden, K.; Parent, J. B.; White, S. L. *Biochim. Biophys. Acta* **1982**, *650*, 209. (c) West, C. M. *J. Cell. Biochem.* **1986**, *32*, 1.

(2) (a) Struck, D. L.; Lennarz, W. J. *The Biochemistry of Glycoproteins and Proteoglycans*; Lennarz, W. J., Ed.; Plenum Press: New York, 1980; pp 35–83. (b) Kornfeld, R.; Kornfeld, S. *Ann. Rev. Biochem.* **1985**, *54*, 631.

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(8) Thiem, J.; Wiemann, T. *Angew. Chem., Int. Ed. Engl.* **1990**, *29*, 80.

(9) An example of the glycosylation of a simple amide has been reported, however, the α-anomer was the predominant product: Kahne, D.; Walker, S.; Cheng, Y.; Van Engen, D. *J. Am. Chem. Soc.* **1989**, *111*, 6881.

(10) Waldmann, H.; Marz, J.; Kunz, H. *Carbohydr. Res.* **1990**, *196*, 75.

(11) To our knowledge one attempt to implement this approach has been reported. The coupling of **2** to several di- and tripeptides was reported, albeit in low (ca. 20%) yield. However, the products were not deprotected. (a) Ishii, H.; Inoue, Y.; Chûjô, R. *Int. J. Pep. Prot. Res.* **1984**, *24*, 422. (b) Ishii, H.; Inoue, Y.; Chûjô, R. *Polymer J.* **1985**, *17*, 695.

(12) A solution of Boc-Asp(α-Bn) (3.0 equiv) and DIEA (5.3 equiv) in DMF was added to crude **2**.[22] (Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP, 3.5 equiv)[20] was added, and the solution was stirred at 23 °C for 17 h. Silica gel chromatography afforded the glycosylated amino acid in 69% yield (none of the α-anomer was detected by ^1H NMR).

(13) Bodanszky, M.; Martinez, J. *J. Org. Chem.* **1978**, *43*, 3071. The rate of succinimide formation is dependent on the identity of the amino acid C-terminal to the aspartate.

(14) The attempted coupling of the peptide H_2N-WDAS-$CONH_2$ with 2–3 equiv of BOP, 16 equiv of DIEA, DMF, 23 °C) afforded the succinimide as the major product by HPLC (30% isolated yield); none of the desired glycopeptide was detected. Additional reactions were not attempted due to the difficulty of the synthesis and purification of this peptide.

(15) Peptides **4**, **7**, **10**, and **12** were synthesized on the Kaiser oxime resin[16,17] using BOP or HBTU couplings, cleaved from the resin with ammonium acetate,[18] and deprotected using trifluoroacetic acid (TFA; **4** and **10**: Asp(tBu) was used) or hydrogenation (**7** and **12**: H_2, Pd on C, DMF, 35–40 psi; Asp(Bn), Tyr(2,6-dichloroBn), Thr(Bn), and Ser(Bn) were used). Ac-WDAS-NH_2[14] was synthesized on the RapidAmide resin using the DuPont RaMPS system, and cleaved and deprotected with TFA/water/ethanedithiol/thioanisole (Asp(tBu) and Ser(tBu) were used). In the cases of peptides **7** and **12**, the corresponding succinimides were formed to varying extents during the synthesis and deprotection. In the most extreme case (**7**), the crude cleavage product contained ~55% succinimide **8** (under certain cleavage conditions, less than 5% of the desired peptide **7** was produced). The structure of **7** was supported by the FABMS fragmentation pattern (NIH MS facility). Succinimide **5** was formed as a byproduct of acetylation of H-DVF-NH_2 (peptide **4** was the major product). Each peptide, peptide succinimide, and glycopeptide was purified to homogeneity by reverse-phase HPLC (C4) and characterized by ^1H NMR and FAB mass spectrometry. The β stereochemistry at the anomeric position of each glycopeptide was confirmed by the magnitude of the coupling constant ($J_{1,2}$ = ca. 9 Hz).

(16) (a) Kaiser, E. T. *Acc. Chem. Res.* **1989**, *22*, 47. (b) Kaiser, E. T., et al. *Science* **1989**, *243*, 187.

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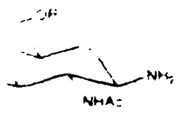
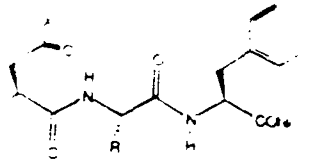
(18) Jarrett, J. T., unpublished results.

Chart I



2 R = Ac

3 R = H

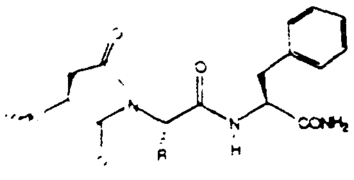


4 R = $CH(CH_3)_2$, X = OH

6 R = $CH(CH_3)_2$, X = NH(GlcNAc)

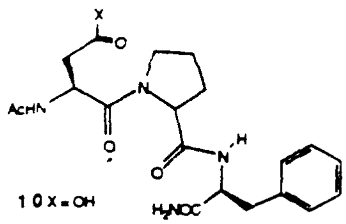
7 R = H, X = OH

9 R = H, X = NH(GlcNAc)



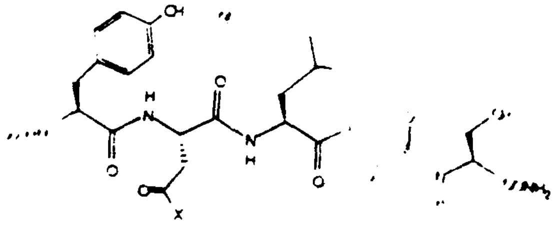
5 R = $CH(CH_3)_2$

8 R = H



10 X = OH

11 X = NH(GlcNAc)



12 X = OH

14 X = NH(GlcNAc)

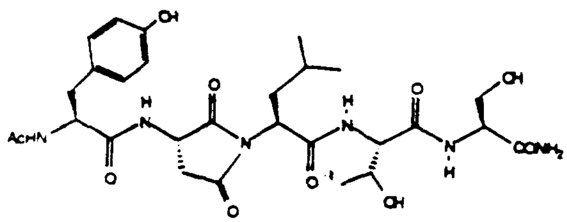


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Table I

entry	peptide[a]	glycosylamine	coupling reagent	DIEA, equiv	product distribution (HPLC),[c] %: glycopeptide	starting material	succinimide
1	4 (DVF)	[illegible]	[illegible] HOBt		95 (6)	0 (4)	5 (5)
2	4 (DVF)	[illegible]	[illegible] HOBt		≤1	≥85 (4)	5 (5)
3	4 (DVF)	[illegible]	[illegible]		20 (6)	75 (4)	5 (5)
4	4 (DVF)	[illegible]	[illegible] HOBt		>90 (6)	5 (4)	<5 (5)
5	4 (DVF)	[illegible]	[illegible] HOBt	[illegible]	90 (6)	0 (4)	10 (5)
6	10 (DPF)	[illegible]	[illegible]	[illegible]	95 (11, 58%)	5 (10)	NA
7	7 (DGF)	3	[illegible] HOBt		80 (9, 53%)	15 (7)	5 (8)
8	12 (YDLTS)	3	[illegible] HOBt		80 (14, 61%)	15 (12, 12%)	5 (13)

[a] ... letter code is used for clarity ... = equiv of glycosylamine ... by absorbance (225 nm) ... was reduced and coupled ... glycosylamine (1.5 equiv of ... V, Val; F, Phe; P, Pro; G, Gly; Y, Tyr; L, Leu; T, Thr; S, Ser; W, Trp; A, Ala. [b] All ... coupling reagent (2 equiv) of HOBt at room temperature, except where otherwise ... yields in parentheses. [d] See footnote 23. In this case, 6 equiv (relative to peptide) ... was premixed with DIEA (3 equiv) and HOBt (3 equiv) for 20 min before ... of BOP.

... which was formed via ... (see the table, entry 3) ... activation with (benzotriazolyloxy)tris(dimethylamino)phosphonium hexafluorophosphate ... conditions[20] ... (BOP)[20] ... of 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (entries 1, 4, 6–8).[21] We have also observed that succinimide formation is directly related to the amount of base present in the coupling reaction (entry 4 vs entry 5); we recommend using the minimum required (2 equiv of glycosylamine or 1 equiv of glycosylamine with 1 equiv of diisopropylethylamine (DIEA)).[22] The choice of the amine component for the

... Preparation of Ac-YDLTS-NH_2 (12 ...) ... GlcNAcNH$_2$ (Sigma ...) ... DMSO 500 μL DMF ... HOBt (50 μmol ...) ... was stirred at 23 °C, and the ... HPLC (C4 analytical column ...) ... H_2O–CH_3CN (0.1% AcOH) ... h, the crude mixture ... 12 H_2O (0.1% AcOH) ... Ac-YD(NAcGlc)LTS-NH_2 (14, 25.0 mg ...) ... (3.8 mg, 12%). Glycopeptide 14 ... FABMS ... calcd 840.4; (M + H)$^+$ = 841.5 ... CONHR, 7.82, d, J = 9 Hz ... stereochemistry ...

[20] Castro, B.; Dormoy, J. R.; Evin, G.; Selve, C. *Tetrahedron Lett.* 1975, 1219.

[21] Knorr, R.; Trzeciak, A.; Bannwarth, W.; Gillessen, D. *Tetrahedron Lett.* 1989, 30, 1927.

[22] Although the model reactions reported in the table involve the use of glycosylamine 3 as nucleophile and as base (2 equiv), we have recently shown that the reaction works equally well (and is more economical) with 1 equiv of 3 and 1 equiv of DIEA as base (other conditions

coupling reaction is also critical. Using our optimized coupling conditions,[19] peptide **4** was coupled to **3** to afford the glycopeptide **6** in good yield (entry 1). However, using the O-acetylated nucleophile **2**,[23] no significant coupling was observed (entry 2). This result, which is consistent with our earlier experience[14] and the low yields obtained in the past using **2** as the amine component,[11] may be due to the decreased nucleophilicity of **2** relative to **3**.[25]

In contrast to peptide **4**, peptide **7** should be optimally disposed for cyclization;[13] however, using our conditions,[19] a 53% yield of glycopeptide **9** was isolated with minimal succinimide formation (entry 7). Glycosylamine **3** has also been coupled to peptides **10** and **12**[26] to provide the glycopeptides **11** (58% purified yield) and **14** (61%), respectively (entries 6 and 8).[15,19] Our current focus is to test the limits of this reaction regarding the size of each component and to adapt this coupling procedure to a solid-phase methodology which allows for selective deprotection of a single carboxyl group at the desired aspartic acid. This procedure[19] has been used to successfully glycosylate resin-bound aspartic acid.[27] Preliminary ^{1}H NMR experiments of glycopeptides **6** and **11** indicate that the attached carbohydrate may influence the conformation of the peptide chain, possibly via the formation of a hydrogen bond.[28] The availability of a wide variety of synthetic glycopeptides will enable us to elucidate these important interactions.

Acknowledgment. We thank Beth Berger for synthesizing and purifying H_2N-WDAS-$CONH_2$[14] and Dr. Chris Warren for his helpful advice. We are grateful to Dr. Ioannis Papyannopoulos of the MIT MS facility (NIH Grant no. RR00317) for FABMS analyses. This work was supported by a grant from the Office of Naval Research (Molecular Recognition Program), the donors of the Petroleum Research Fund, administered by the American Chemical Society, and funds from the Camille and Henry Dreyfus Foundation (New Faculty Award) and Merck & Co. (Faculty Development Award).

(23) A method for the conversion of peracetylated oligosaccharides with GlcNAc at the reducing terminus to the β-glycosylamine and subsequent coupling to an amino-protected aspartic acid ester has been reported.[7] We have modified that procedure to minimize handling of the unstable glycosyl amine as follows: the β-glycosylazide[7b,5] was treated with 1,3-propanedithiol[24] (5 equiv) and diisopropylethylamine (3 equiv) in dimethylformamide (DMF) for 1.5 h at 23 °C to afford the β-glycosylamine **2**. Solvent was removed in vacuo, and the crude product was coupled directly.[12]

(24) Bayley, H.; Standring, D. N.; Knowles, J. R. *Tetrahedron Lett.* **1978**, 3633.

(25) A referee suggests that the observed difference in yield may simply be due to the lability of **2** under the reaction conditions. Although the rearrangement (see ref 7b) and dimerization (Paul, B.; Korytnyk, W. *Carbohydr. Res.* **1978**, *67*, 457) of **2** are precedented, we feel that this explanation is unlikely in light of the successful coupling of **2** to Boc-Asp(α-Bn) (see ref 12).

(26) Peptide **12** is derived from the glycosylation site of ovalbumin. Glabe, C. G.; Hanover, J. A.; Lennarz, W. J. *J. Biol. Chem.* **1980**, *255*, 9236.

(27) Fluorenylmethoxycarbonyl (Fmoc) protected aspartic acid bound to the polystyrene-based methylphenacyl resin[29] was treated with **3** (2 equiv), HBTU (3 equiv), and HOBt (1 equiv) in DMF/DMSO. After shaking for 25 h, the resin was photolyzed (350 nm, DMF/2 equiv of H_2O, 23 h, 23 °C) to provide the product Fmoc-Asn(GlcNAc), as well as some unreacted Fmoc-Asp (~4:1 glycoamino acid to starting material, by HPLC).

(28) Glycopeptides **11** and **6** were analyzed (^{1}H NMR, 300 MHz, DMSO) over the temperature range 20–50 °C. For **11**, the chemical shifts of two amide protons were relatively insensitive to temperature ($\Delta\delta/\Delta T \leq 3.5$ ppb/deg), indicating the participation of these protons in hydrogen bonds.[11] For **6**, one amide proton appears to be involved in hydrogen bonding. Details of these and other NMR experiments will be published elsewhere.

(29) Wang, S. S. *J. Org. Chem.* **1976**, *41*, 3258.